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EFFECT OF HYPERBARIC CARBON DIOXIDE ON SPORES AND VEGETATIVE CELLS OF *BACILLUS STEAROTHERMOPHILUS*

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Spore suspensions in water, 0.05M pH 7 phosphate buffer, 0.05M pH 4.5 and 0.05M pH 4.0 acetate buffer, and antibiotic assay media supplement broth (AAMS) were subjected to 50 psi to 1100 psi carbon dioxide at various temperatures in Parr bombs for up to 96 hours. Spores were resistant to high pressure carbon dioxide treatment at each pH and at both high and low temperatures. Spore suspensions that were first heat activated and then incubated at 55^{0}C for a short time increased sensitivity to destruction by CO_2. Although spores were resistant to destruction by hyperbaric CO_2 low pressure (50 psi) CO_2 was seen to completely inhibit spore germination and outgrowth. Inhibition to germination was found to be reversible. Spore germination was found to be impaired in carbonated AAMS broth. However, the addition of 50 mM $NaHCO_3$ to AAMS enhanced germination and outgrowth.

Vegetative cells were shown to be sensitive to CO_2 treatment. Complete destruction of 10^6 cells in AAMS broth was obtained by exposure to 400 psi CO_2 for 1.5 hours.

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PREFACE

This study was conducted during the months of May through August 1992, by Dr. Chester Roskey , Framingham State College, Framingham, MA, under the general supervision of Dr. Anthony Sikes (project officer), U. S. Army Natick Research, Development and Engineering Center, Natick, MA. The research was sponsored by the U.S. Army Research Office of Scientific Services Program administered by Battelle (Delivery Order 259, Contract No. DAAL03-91-C-0034). The work was funded under the project titled "Hyperbaric Preservation," DA project AH5240D00.

Dr. Roskey's research was designed to ascertain the following: (1) determine the effects of pressurized CO_2 on the microbial activity of the thermophilic spoilage bacterium, Bacillus stearothermophilus, (2) evaluate the effects of gas mixtures, e.g., CO_2:O_2:N_2, at different partial pressures on both vegetative and sporeforming bacteria in model food systems and ration items, (3) select optimum atmosphere/pressures as a result of time-temperature studies and (4) apply optimum condition to test potential of shelf life extension of perishable foods.

Effect of Hyperbaric Carbon Dioxide on Spores and Vegetative Cells of *Bacillus stearothermophilus*.

Introduction

Carbon dioxide inhibits the growth of many microorganisms. This fact has been exploited in preventing bacterial food spoilage through modified atmosphere packaging (MAP). The ability of high concentrations of CO_2 to retard the growth of spoilage flora in meat, poultry, and fish is well documented (1, 3, 17, 18, 20, 21).

The inhibitory effect of CO_2 increases when it is applied under pressure (3, 8, 10, 11, 15,). Enfors and Molin reported that germination of *C. sporogenes* was inhibited slightly at 4 atmospheres and almost completely at 10 atmospheres, whereas germination of *C. perfringens* was slightly stimulated at 4 atmospheres, unaffected at 10 atmospheres and stopped at 25 atmospheres of pure CO_2 (10). Doyle revealed that at atmospheric pressure 100% CO_2 delayed toxin production by *C. botulinum*. Increasing the pressure to 8.8 atmospheres caused a further delay in toxin production. Pressurized CO_2 was also lethal to *C. botulinum* with the viability decreasing two log cycles during eight weeks of storage under 8.8 atmospheres. Loss of viability was directly related to pressure of CO_2 and length of exposure (8). Haas et al, investigated the effect of hyperbaric CO_2 on food microbiota and found that the total count of microorganisms in foods can be reduced by treatment with CO_2 under pressure. These workers determined that 900 psi CO_2 had the greatest effect and that inhibition decreased with decreasing water activity. They also reported that low pH acted synergistically with CO_2 and that microbicidal activity was enhanced at higher temperature (15). However Blickstad et al, had reported earlier that the inhibitory effect of CO_2 was greater at lower temperatures (3).

The capacity of the almost inert spores to spoil food depends on their ability to germinate, outgrow and achieve extensive vegetative multiplication in the food. Interruption of this chain

of events at any point will prevent spoilage. Bacillus stearothermophilus (BS) is a ubiquitous sporeformer. As a thermophilic sporeformer it is of particular concern as a potential spoilage microbe in thermally-processed foods. The bacterium can grow under both aerobic and anaerobic conditions, and ferments sugars (2). Spores of BS may be present in "commercially sterile" foods and may be responsible for "flat-sour" spoilage of low acid foods (14).

The objectives of the present study were to investigate the effect of hyperbaric CO_2 on survival and growth of vegetative cells of BS as well as its effect on viability, germination, and outgrowth of endospores of BS.

METHODS AND MATERIALS

Test bacteria

Bacillus stearothermophilus ATCC 12980 (Type strain). Vegetative cells were maintained on Antibiotic Assay Media Supplement (AAMS) agar slants at 4°C. Vegetative cells were cultivated in AAMS broth at 55°C. Spore suspensions of BS were provided by A. Sikes of the U.S. Army Natick Research, development and Engineering Center. Spore suspensions were prepared as described by Feeherry et al (13).

Hyperbaric treatment

Cells or spores were suspended in either sterile distilled deionized water, 0.05M phosphate buffer pH 7.0, 0.05M acetate buffer pH 4.5, 0.05M acetate buffer pH 4.0, or AAMS broth, in 16 mm by 150 mm screw-capped culture tubes. The caps were loosely secured to permit free gas exchange. The tubes were placed in the cylinder of 1850 ml Parr cell disruption bombs (Parr Instrument Company, 211 Fifty-Third Street, Moline, Illinois 61265 USA). After loading, the cylinders were sparged with either nitrogen or carbon dioxide at 200 psi for 1 minute to remove air. The cylinders were then pressurized (50 psi-1000 psi) and then stored at various temperatures (3°, 25°, 55°, 65°, 75°, 90°, and 100°C) for 1 to 96 hours.

Activation of spores

Spores were activated by subjecting suspensions in water or AAMS broth to flowing steam for 15 minutes. Some spore suspensions were activated by suspension in 9% or 90% v/v aqueous dimethylsulphoxide (DMSO).

Preparation of germinated spores

Heat-activated spores suspended in AAMS broth were incubated for 3 hours at 55°C.

Carbonation

Sixty ml of ice-chilled AAMS broth were aseptically transferred to an iced sterile 250 ml cylinder. A sterile 10 ml pipette was fitted to the regulator hose of a CO_2 tank. The pipette was placed into the broth and CO_2 bubbled through the media at 50 psi for 4 minutes. The pH of the carbonated AAMS broth was 5.44.

Effect of sodium bicarbonate

A 5% $NaHCO_3$ solution was prepared and filter sterilized. One ml of the filter-sterilized solution was added to each of three tubes containing 10 ml of AAMS broth.
One ml of sterile water was added to each of three tubes containing 10 ml of AAMS as a control. Each of the six tubes was inoculated with 0.1 ml of a BS spore suspension. All tubes were then incubated at 55°C for 24 hours. The pH of the $NaHCO_3$ supplemented broth was 7.6.

UV absorption spectra

One hundred ml of AAMS broth was inoculated with 1.0 ml of an overnight culture of BS in AAMS and then incubated for 7 hours at 55°C. Cells were harvested by centrifugation at 1000 X g for 20 minutes. Harvested cells were washed three times in phosphate buffer and then resuspended in buffer.

Cell suspensions were then subjected to 850 psi CO_2 for 17 hours at 25°C. Cell suspensions similarly prepared were stored under ambient conditions for the same time as controls. After incubation the CO_2 treated and control cultures were centrifuged at 1000 X g for 30 minutes and the UV absorption spectrum of the supernates was determined. UV absorption analysis was provided by K. Whitburn (U.S. Army Natick Research, Development and Engineering Center).

Enumeration

Cells or spores were enumerated on AAMS agar spread plates. Dilutions were made in either water or 0.05 M phosphate buffer pH 7.0. All dilutions were made with a Gilson pipetman P200 or P1000 fitted with appropriate tips. All counts reported represent the mean of duplicate or triplicate determinations.

RESULTS AND DISCUSSION

Effect on vegetative cells

Hyperbaric CO_2 was shown to have a biocidal effect on vegetative cells of BS. Destruction of vegetative cells was shown to be dependent on both time of exposure, as well as CO_2 pressure. The effect of a 1 hour exposure to CO_2 at 0, 300, 400 and 500 psi at 25°C is shown in Figure 1. Carbon dioxide treatment for 1 hour at 400 and 500 psi resulted in more than a two log cycle reduction in viability of *B. stearothermophilus* cells (from log 6.7 to log 4.2). Control cells (log 6.7) at ambient conditions did not decrease in viability in this interval. There was no apparent difference in lethality due to increasing CO_2 pressure during the 1 hour treatment period, when the CO_2 pressure was 300 psi.

Extending the exposure period, at a lower pressure (200 psi), beyond 1 hour resulted in a further decline in viability of vegetative cells. The effect of exposure time on survival of vegetative cells exposed to 200 psi over a 3.5 hour period was greater than 400 psi for only 1 hour as depicted in Figure 2. The data indicates that increasing exposure time can result in increasing lethality at lower pressures. The population of vegetative cells decreased over four log cycles (log 6.3 to log 2.0) due to 200 psi CO_2 treatment for 3.5 hours. The data presented in Figure 3 indicated that complete destruction of vegetative cells was achieved through hyperbaric CO_2 treatment at 400 psi by increasing the time from 1 h (Fig. 1) to 1.5 h. Nitrogen gas at the same pressure had no adverse effect on viability of BS vegetative cells.

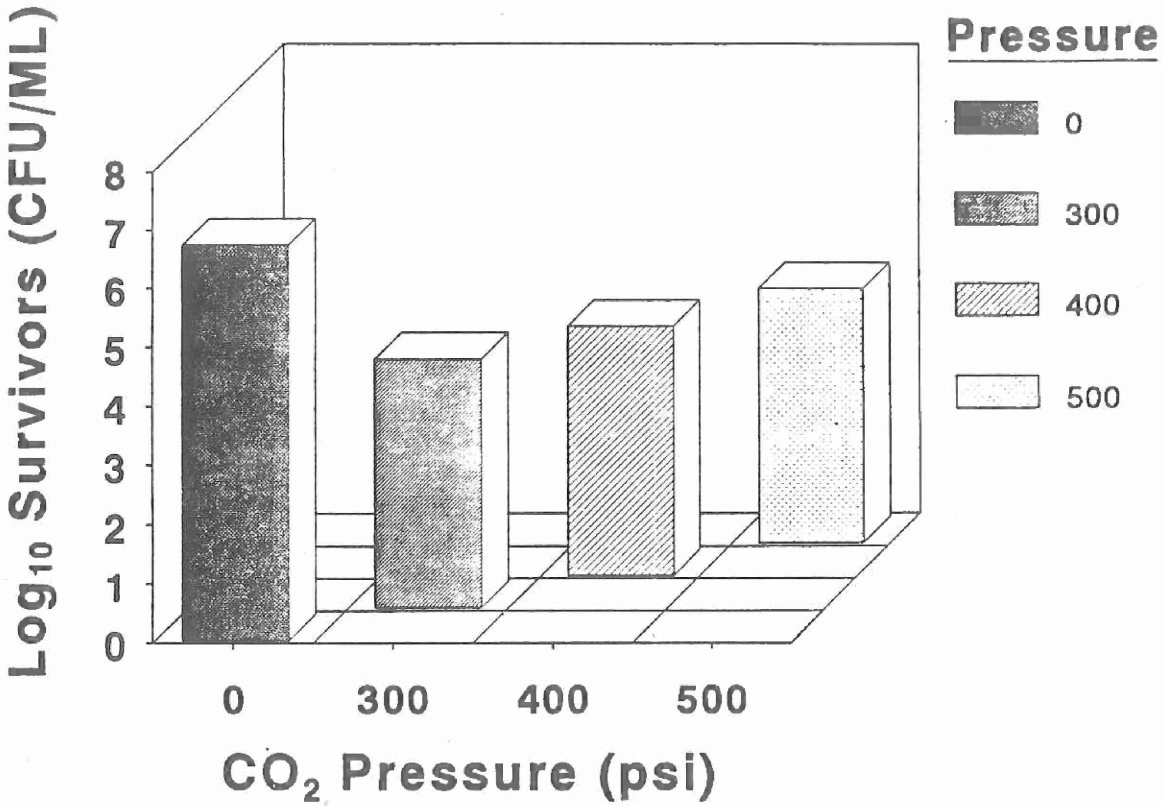

Figure 1. Effect of exposure to 300, 400, and 500 psi CO_2 for one hour at 25°C on survival of vegetative cells of Bacillus stearothermophilus.

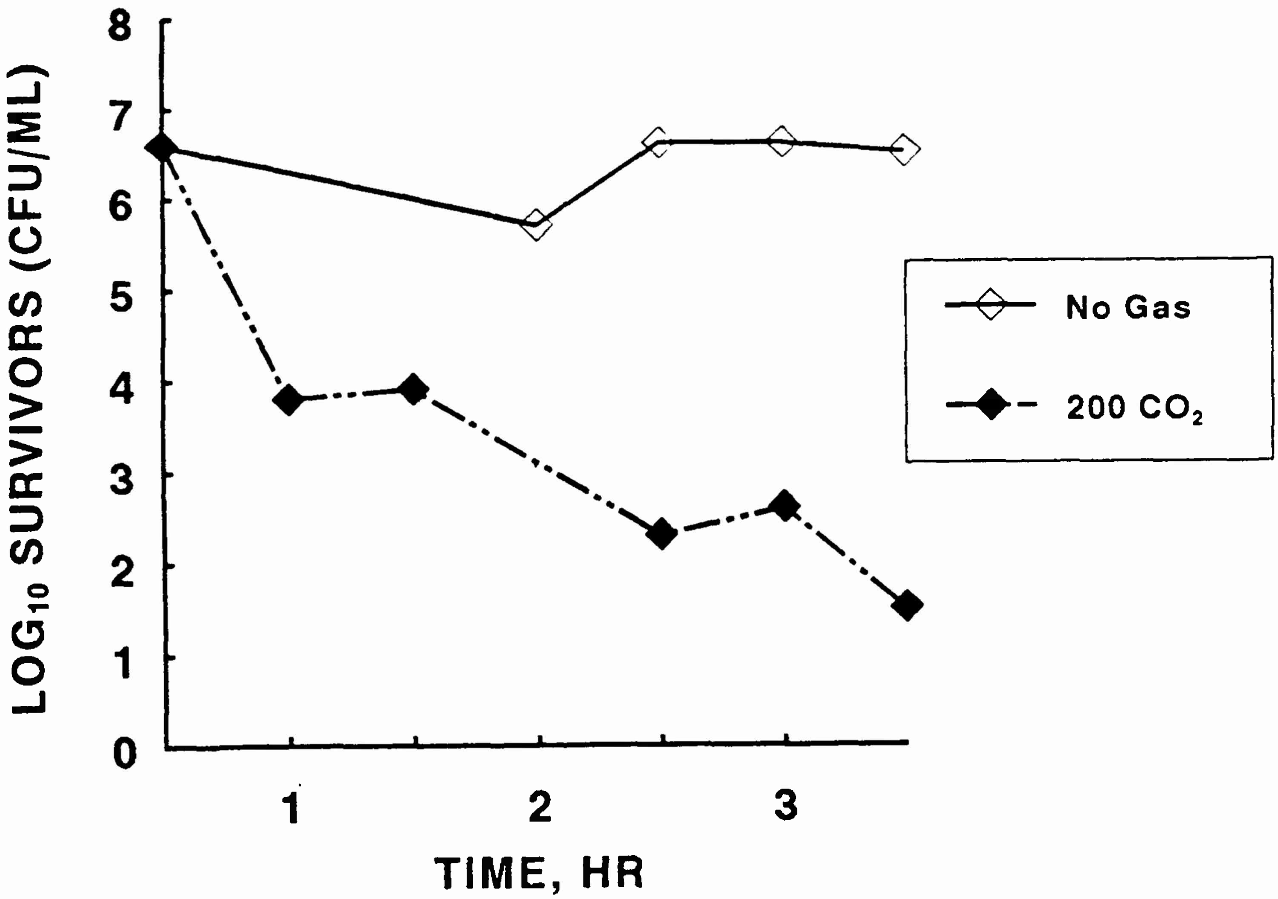

Figure 2. Effect of exposure to 200 psi CO_2 for 3.5 hours at 25°C on survival of vegetative cells of <u>Bacillus stearothermophilus</u>.

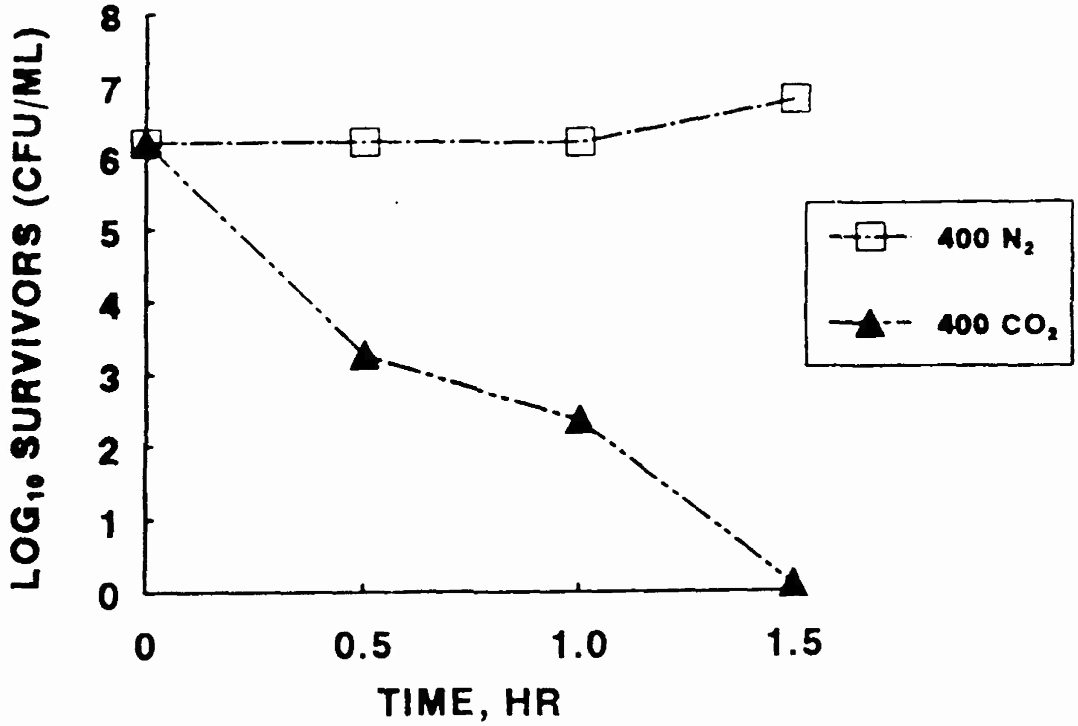

Figure 3. Effect of exposure to 400 psi CO_2 or N_2 at 25°C on survival of vegetative cells of *Bacillus stearothermophilus*.

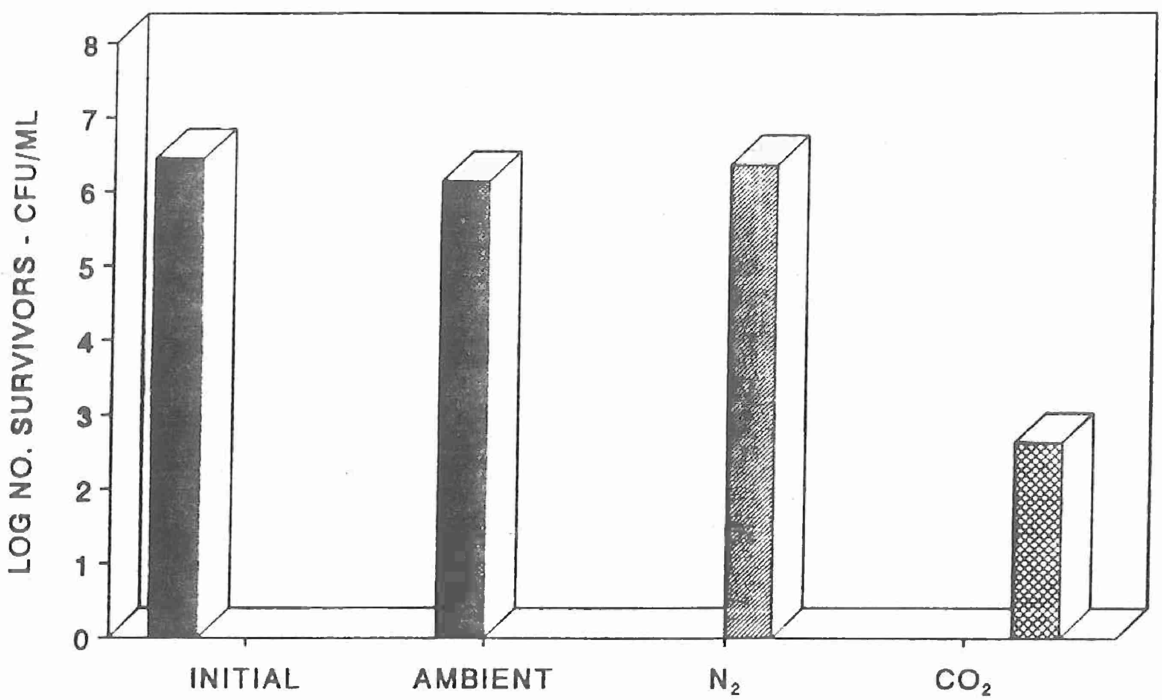

Figure 4. **Effect of exposure to 875 psi CO_2 and N_2 at 25°C for 1.25 hours on survival of vegetative cells of *Bacillus stearothermophilus*.**

Since complete destruction of _B_. _stearthermophilus_ vegetative cells culture was attained by exposure to 400 psi CO_2 for 1.5 hours, one would expect that higher pressures could achieve the same effect in less time. However, when vegetative cells were subjected to 875 psi CO_2 for 1.25 hours at room temperature (Figure 4) there was only a four log cycle reduction in vegetative cells (log 6.45 to log 2.63). Viable cells were still recovered. Cells subjected to N_2 at 875 psi and cells kept under ambient conditions for the same period were unaffected.

These results are in agreement with those of other workers (1, 3, 7, 15,17) who reported that carbon dioxide under pressure was lethal to vegetative cells of food spoilage microorganisms, and rate of lethality was dependent on the amount of pressurized CO_2 and the length of exposure. Haas et al (15) found that all microorganisms on chives, thyme, parsley, mint, and spoiled apple juice were killed by a 30-minute exposure to 800 psi CO_2 at elevated temperature (45°C). In the present study, _B_. _stearothermophilus_ vegetative cells were not completely destroyed by a 75 minute exposure to 875 psi at a lower temperature (25°C). Survival of the _B_. _stearothermophilus_ treated cells in this study may be attributed to the lower temperature treatment. Haas et al. (15) reported that higher temperatures enhanced the bactericidal effect of CO_2. Enfors and Molin (12), on the other hand, showed that there was greater CO_2 inhibition of the growth rate of _Pseudomonas fragi_ and _Bacillus cereus_ at lower temperatures.

Effect on endospores

Spores of _B_. _stearothermophilus_ proved to be remarkably resistant to hyperbaric CO_2 treatment. The effect of CO_2 and N_2 on the survival of BS spores under varying conditions of temperature and pH is shown in Table 1.

When _B_. _stearothermophilus_ spores at a population density of ca. log 4.7 were suspended in distilled water, 0.05M acetate buffer pH 4.0, 0.05M acetate buffer pH 4.5, or 0.05M phosphate buffer pH 7.0, and then subjected to hyperbaric CO_2 for 1 to 96 hours, there was no apparent spore destruction. Aliquots of the same spore suspensions subjected to ambient gas conditions and those subjected to hyperbaric N_2 were similarly unaffected.

There is agreement among workers regarding the effect of temperature on the bactericidal activity of CO_2. Blickstad et al, (3) found that CO_2 inhibited the microbial flora of pork to a greater extent at 4°C than at 14°C. Daniels et al (6) attributed increased inhibition by CO_2 at lower temperature to increased solubility of the gas in the water phase. However, higher temperatures enhanced the microbicidal action of CO_2 (15). The results obtained during the present investigation revealed that neither high (95°-100°C) nor low temperature (3°C) had any effect on the sporicidal activity of hyperbaric CO_2.

Blocher and Busta (4) stated that the heat resistance (D-value) of spores decreased as the pH of the medium decreased. Haas et al (15) reported that lower pH acted synergistically with CO_2 pressure. These workers found that 800 psi CO_2 at 70°C killed spores of _Clostridium sporogenes_ suspended in thioglycolate broth when the pH of the broth was between 2.5 and 3.0, but did not kill spores when the pH of the broth was 4.0.

Table 1. The effect of hyperbaric carbon dioxide on viability of *Bacillus stearothermophilus* spores.

pH	Temp. °C	Pressure psi	Time hrs	Survival Fraction log $N_{CO2}{}^{b}/N_0{}^{a}$	log $N_{A^c}/N_0{}^{b}$	log $N_{N2}{}^{d}/N_0$
4	3	800-550	68	0.98	0.98	
4	25	840-890	68	0.94	0.93	
4	55	840-1050	68	0.99	0.87	
4	65	800-900	45	0.98	0.86	0.82
4.5	3	820-540	96	1.04	1.06	1.06
4.5	25	830-850	96	1.01	0.98	
4.5	65	840-900	23	0.99	0.82	0.89
4.5	90	850-875	22	0.55	0.78	
7	25	800-930	23	1.01	0.96	1.01
7	55	940-1050	46	1.01	0.80	0.81
7	65	940-975	20	1.00	0.81	
7	75	875-1000	26	1.06	0.91	
7	100	900-1100	1	1.09	1.08	

[a]N_0 = initial population (mean initial population was log 4.7)

[b]N_{CO2} = number present after CO_2 treatment.

[c]N_A = number present after exposure to ambient gas atmosphere for the treatment period.

[d]N_{N2} = number present after exposure to N_2 gas at the same pressure as CO_2 for the same time.

Examination of the data presented in Table 1 reveals that spores were able to tolerate exposure to hyperbaric CO_2 for 1-96 h when suspended in buffer at pH 4.0, pH 4.5, and pH 7.0. However, high temperature (90°C) and low pH (4.5) were found to act synergistically with hyperbaric CO_2 to reduce viable spores. Figure 5 shows that 850-875 psi CO_2 treatment over a 22 hour period resulted in nearly a two log cycle reduction in viability of spores (log 4.34 to log 2.40). The viability of spores held at ambient gas conditions at pH 4.5 and 90°C was also diminished by the treatment (log 4.34 to log 3.40) but to a lesser extent. Additional studies should be done to more clearly characterize the synergistic effects of high temperature, low pH, and hyperbaric CO_2.

Since unactivated spore suspensions proved to be resistant to destruction by hyperbaric CO_2 treatment, attempts were made to evaluate the effect of hyperbaric CO_2 on spores that had undergone "activation." Accordingly, spore suspensions in 0.05M phosphate buffer, pH 7.0 were activated by subjecting the suspensions to flowing steam for 5, 10, 15, 20, and 25 minutes. Viable counts were made immediately before and after steam activation and after exposure to 800-875 psi CO_2 at 25°C for 21 hours. The effect of heat activation on survival of spores exposed to hyperbaric CO_2 is shown in Table 2. Examination of the data in Table 2 reveals that hyperbaric CO_2 treatment was not effective in reducing the viability of heat activated B. stearothermophilus spores.

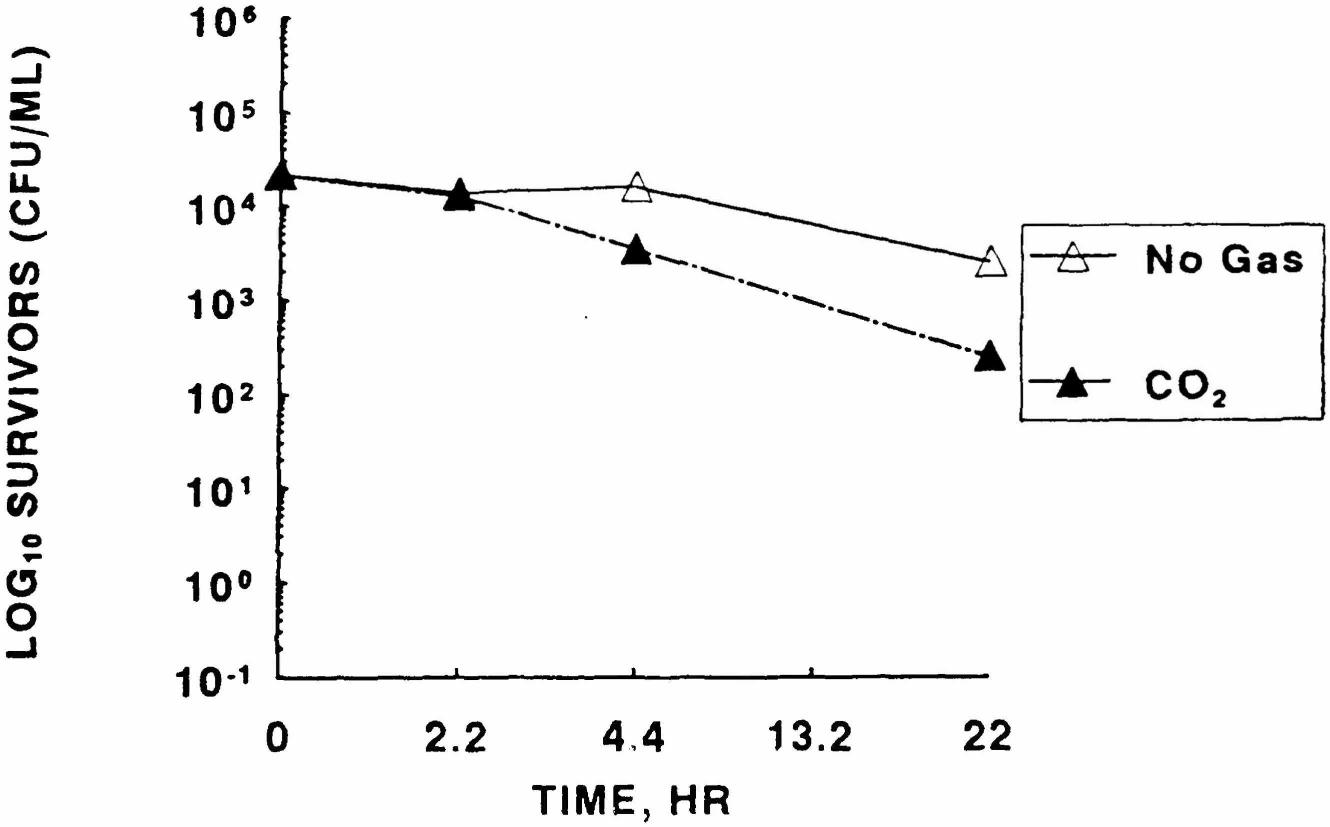

Figure 5. Effect of exposure to 850-875 psi CO_2 at 90°C on viability of <u>Bacillus stearothermophilus</u> spores suspended in 0.05M acetate buffer pH 4.5.

Table 2. The effect of heat activation on survival of Bacillus stearothermophilus spores exposed to 800-875 psi CO_2 for 21 hours at 25°C.

Time in steam (minutes)	CFU/ml[a] after steam treatment	CFU/ml after CO_2 treatment	% Reduction
0	46.8×10^3	32×10^3	0
5	7.1×10^3	30×10^3	0
10	13.6×10^3	20×10^3	0
15	24.3×10^3	25.5×10^3	0
20	23.8×10^3	22.5×10^3	5.5
25	26.4×10^3	20.5×10^3	22.0

[a] CFU = colony forming units

The maximum reduction was only 22.0 % of spores activated for 25 min. However, activation treatment of B. stearothermophilus for 60 minutes at pH 2.0 and 60°C showed a marked decrease in their heat resistance (9). Additional studies to evaluate the effect of hyperbaric CO_2 on heat activated BS spores at low pH may prove fruitful.

Widdowson (22) reported that reduction of the S-S linkages in spore protein may lead to rapid activation. It was found that pretreatment of a spore suspension with 90% v/v aqueous solution of dimethylformamide produced rapid activation of B. pantothenticus spores even at 4°C.

Widdowson (22) also reported that dimethylsulphoxide (DMSO) was effective in producing rapid activation at room temperature. She suggested that the decrease in the amount of H-bonding in the spore brought about by these compounds might be expected to result in an increase of sensitivity to damaging agents. With this in mind, an experiment was designed to assess the effect of hyperbaric CO_2 on DMSO activated spores. Spore suspensions were made in water, 9% v/v aqueous DMSO, and in 90% aqueous DMSO. Viable spore populations were determined init.ally and after 20 hours at 25°C under both ambient and hyperbaric (900 psi) conditions. The effect of CO_2 on DMSO activated spores is shown in Table 3. The data presented reveals that spores activated by suspension in DMSO were not more susceptible to destruction by exposure to hyperbaric CO_2 than were spores suspended in water.

There was no apparent difference between the survival of B. stearothermophilus spores treated by exposure to 900 psi for 20 hours at 25°C and controls that were exposed to ambient air under the same conditions.

Since spore suspensions as well as "activated" spore suspensions proved to be resistant to hyperbaric CO_2 treatment, activated spore suspensions were incubated (in AAMS and water) at 55°C for 3 hours in order to allow time for germination prior to being subjected to hyperbaric CO_2.

The effect of heat activation and incubation for three hours at 55°C on the survival of heat activated and germinated B. stearothermophilus spores to 900 psi CO_2 for 67 hours at 25°C is shown in Table 4.

Table 3. The effect of DMSO activation on survival of *Bacillus stearothermophilus* spores exposed to 900 psi CO_2 for 20 hours at 25°C.

Spores suspended in	Initial count CFU/ml	% Survival after 20 hours at 25°C CFU/ml	
		Ambient	CO_2
H_2O	100×10^3	28	19
9% DMSO	75×10^3	44	33
90% DMSO	54×10^3	76	91

Germination and growth of spores resulted in a 100 fold increase in viable counts on AAMS. Carbon dioxide treatment of the activated and germinated spore suspension resulted in a two log cycle reduction in the population. Heat activated and germinated spores that were subjected to ambient gas conditions for the same period did not exhibit a similar decrease in viability. Spores suspended in water, heat activated, and incubated, responded as did spores suspended in AAMS broth. Although there was no evidence of germination and outgrowth of the spores in the water suspension, the spores nevertheless were susceptible to destruction by hyperbaric CO_2.

Table 4. The effect of heat activation and incubation for three hours at 55°C on survival of Bacillus stearothermophilus spores exposed to 900 psi CO_2 for 67 hours at 25°C.

Spore Suspension in	Initial count CFU/ml	Count after 3 hours at 55°C CFU/ml	Count after 67 hours at 25°C CFU/ml	
			Ambient	CO_2
AAMS[a]	40×10^3	61×10^5	28×10^5	73×10^3
H_2O	48×10^3	18×10^3	11×10^3	71×10^1

[a] AAMS = antibiotic assay medium supplement

Effect of hyperbaric CO_2 on germination and outgrowth

The experimental work reported has shown that spores are virtually indestructible to exposure to CO_2 under the conditions tested. An experiment was undertaken to determine the effect of CO_2 on germination and outgrowth of BS spores in AAMS broth. The results of this study are shown in Table 5. Three replicates of each sample were prepared. Initially, each of the 12 tubes contained ca. 29×10^3 CFU/ml of BS spores. After 18 hours at 55°C, all tubes stored under ambient gas conditions were turbid. All tubes that were incubated under 1000 psi CO_2 were clear. Carbon dioxide at 1000 psi had prevented the germination and outgrowth of BS spores. When the spore suspensions that had received CO_2 treatment underwent further incubation under ambient conditions at 55°C, all these tubes became turbid.

Table 5. The effect of 1000 psi CO_2 on activated and non activated spores of _Bacillus_ _stearothermophilus_ suspended in AAMS broth and stored at 55°C for 18 hours.

Spore suspension in AAMS broth	Appearance of broth after 18 hours at 55°C	
	Control Ambient	Treated CO_2 1000 psi
Heat Activated	Turbid	Clear
Not Activated	Turbid	Clear

Although CO_2 had prevented germination and outgrowth of spores, it did not kill them or impair the spores ability to undergo further development under more favorable ambient conditions.

Additional tests were performed to ascertain the lowest CO_2 pressure that would prevent germination and outgrowth of BS spores. Triplicate tubes of AAMS broth were inoculated with ca. 10^3 spores and then stored at 50 psi, 75 psi, 100 psi, 200 psi, 400 psi, and 600 psi CO_2. Triplicate tubes were also inoculated and stored under 50 psi and 600 psi nitrogen. All samples were incubated for from 16 to 87 hours at 55°C. All samples that were treated with CO_2 remained clear while all controls (33 tubes) that were incubated concurrently under ambient conditions became turbid. Samples that were incubated at 55°C under a nitrogen atmosphere (50 psi or 600 psi) behaved as did ambient controls.

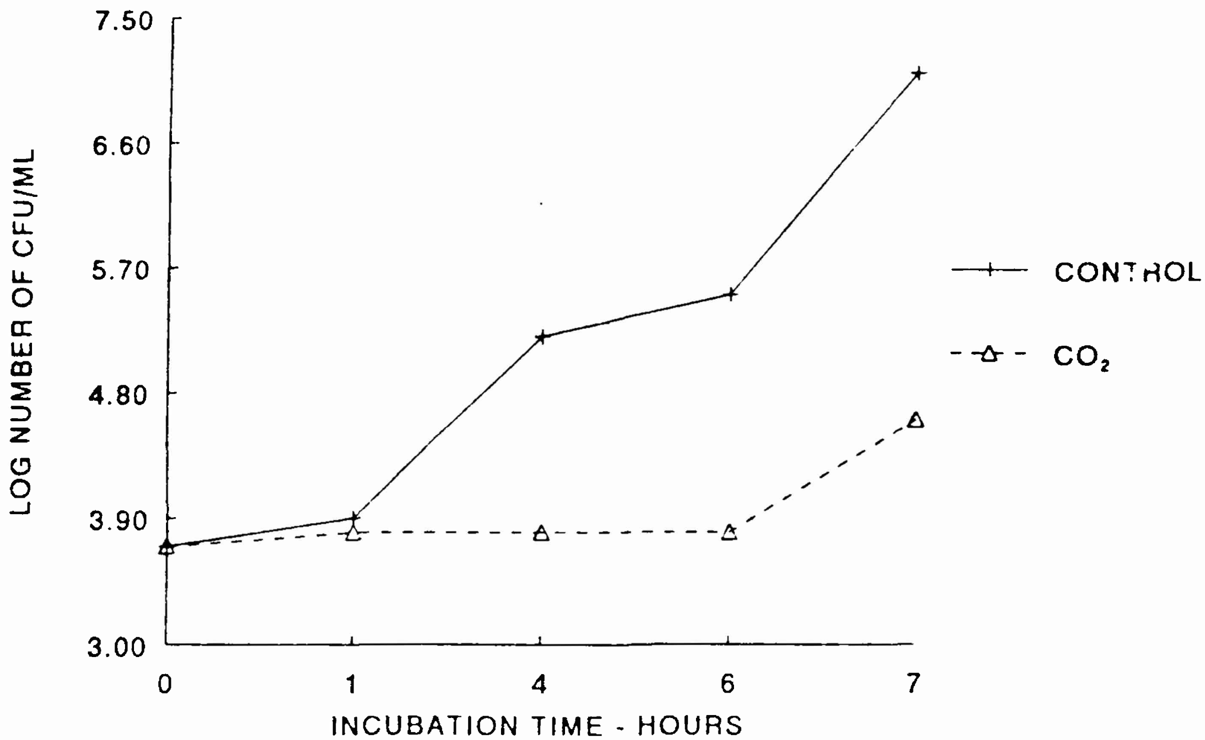

Figure 6. **Effect of carbonation on germination and outgrowth of <u>*Bacillus*</u> <u>*stearothermophilus*</u> spores in AAMS broth during incubation at 55°C.**

When BS spores were suspended in carbonated AAMS broth and incubated at 55°C, growth and germination were adversely affected. The effect of carbonation on the growth of BS spores at 55°C is depicted in Figure 6. The data obtained indicate that carbonation of the media completely prevented germination and outgrowth for 6 hours. After 6 hours the spores overcame the inhibitory effect. Carbonation of the media caused the pH to drop from 6.7 to 5.4. Inhibition of germination may be due to the lower pH of the media. However, vegetative growth of BS is reported to be rapid in many foods of pH above 5.0 if held at an elevated temperature (2). Blocher and Busta (4) reported that the minimum pH for germination of BS spores at 55°C is less than 5.4.

Cross et al (5) reported that germination of four strains of Bacillus in a yeast-dextrose broth medium was inhibited by bicarbonate at 5, 25, and 50 mM concentration. Similar findings were reported by Hachisuka et al, who reported that 50mM $NaHCO_3$ inhibit germination of Bacillus subtilis spores (16). The effect of 54 mM $NaHCO_3$ on germination and outgrowth of BS spores in AAMS broth was evaluated and the results are shown in Figure 7. The BS spores were not inhibited by bicarbonate, but rather growth and germination were enhanced. Enhancement of germination may be attributed to the buffering capacity of the $NaHCO_3$. The pH of the bicarbonate supplemented media was 7.0 after incubation, while the pH of the unsupplemented media was 4.7. The data suggests that $NaHCO_3$ be added to AAMS routinely to optimize cultivation of BS.

UV absorption spectra of supernates of CO_2 treated and control BS vegetative cells.

Shigehisa et al (19) reported that high hydrostatic pressure (3000-6000 atm) killed all microorganisms tested except Bacillus cereus spores.

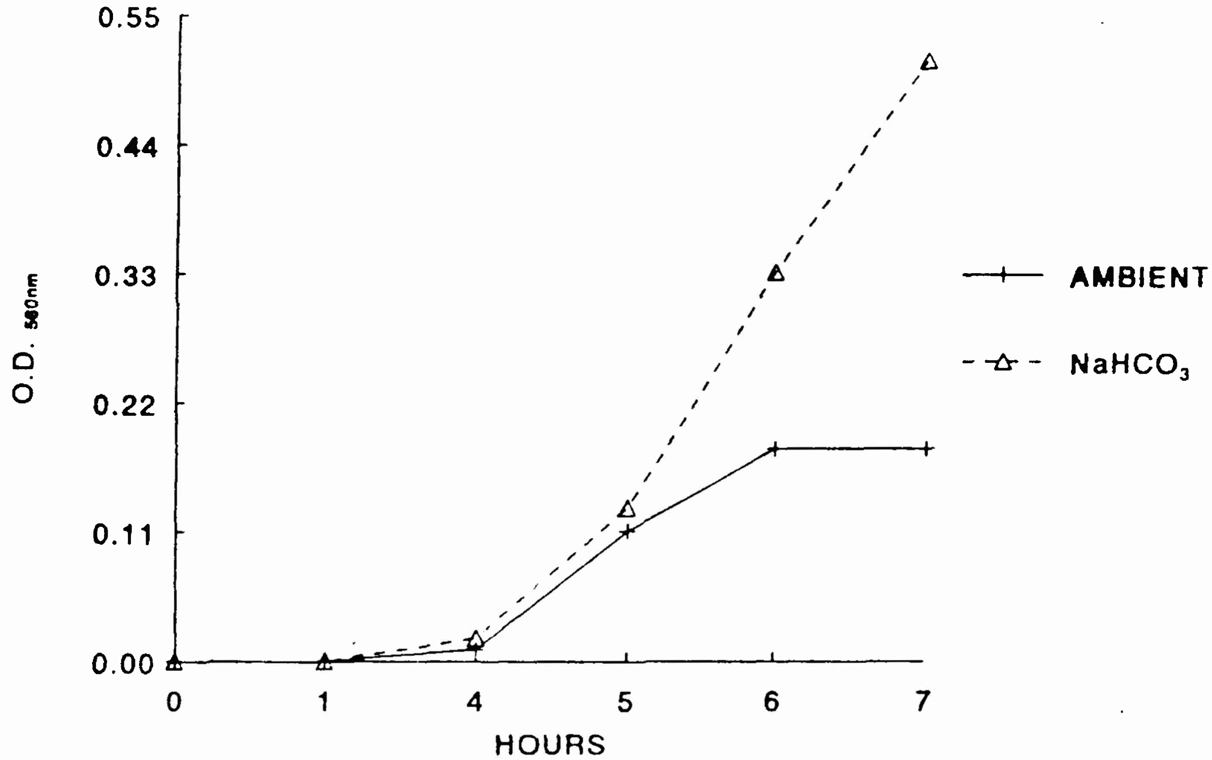

Figure 7. Effect of 54 mM $NaHCO_3$ on germination of Bacillus <u>stearothermophilus</u> spores in AAMS during incubation at 55°C.

Examination of the supernatant fluid of E. coli cells subjected to such hydrostatic pressure revealed they leaked cytoplasmic RNA that was detected by analysis of the UV absorption spectrum of the culture supernate. Supernates obtained from vegetative cells that had been subjected to 850 psi CO_2 for 17 hours, as well as supernates obtained from control cells, were analyzed by UV spectroscopy. Results of this analysis are shown in Figure 8. The UV spectra revealed no significant difference in the amount of DNA/RNA present in the supernatant fluid of ambient and CO_2 treated cells. However, there was a greater absorbance in the 210-240 nm range in the CO_2 treated than in the ambient sample supernate (19). Further analysis by gas chromatograpy of the nature of the molecules absorbing in the 210-240 nm range may reveal information regarding the nature of the lesion induced by hyperbaric CO_2 on vegetative cells.

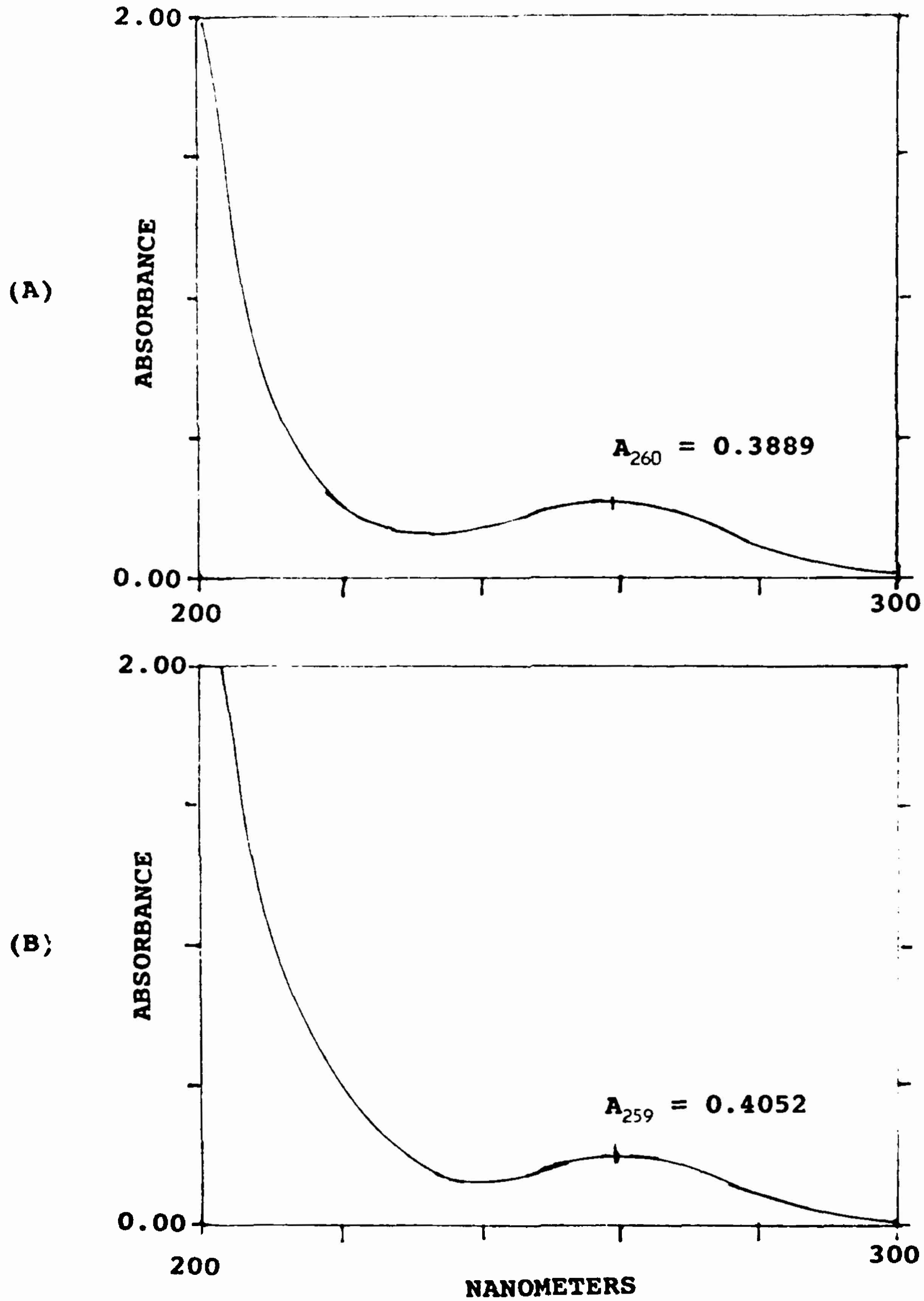

Figure 8. Ultraviolet absorption spectra of culture supernates obtained from:
(A) BS vegetative cells in phosphate buffer, pH 7.0 for 17 hours at 25°C under ambient air.
(B) BS vegetative cells in phosphate buffer, pH 7.0 for 17 hours at 25°C under 850 psi CO_2.

CONCLUSIONS

The objectives of the present study were to determine the effect of hyperbaric carbon dioxide on survival and growth of vegetative cells of Bacillus stearothermophilus as well as its effect on viability, germination and outgrowth of spores.

Vegetative cells were found to be sensitive to damage by relatively low pressure CO_2 treatment for a short time. Complete destruction of 10^6 cells in AAMS broth was obtained by exposure to 400 psi CO_2 for 1.5 hours.

Spores, however, were found to be unaffected by high pressure carbon dioxide treatment. Spores remained viable after exposure to 800 - 1100 psi CO_2 for as long as 96 hours. Spores survived CO_2 treatment at low (3°C) as well as high (100°C) temperatures. Spores survived CO_2 treatment at pH 4, 4.5, and 7. However, data obtained suggests that high pressure (850-875 psi) CO_2 exposure at high temperature (>90°C) act synergistically to compromise spore viability.

Spores that had undergone a heat activation followed by a 3 hour incubation at 55°C were susceptible to destruction by CO_2. Germination was not necessary for susceptibility to CO_2, for heat activated spores suspended in water were as readily destroyed by CO_2 exposure as were those suspended in a rich nutrient media.

Carbon dioxide at low pressure (50 psi) inhibited the germination and outgrowth of spores. The inhibition was found to be reversible. Spores that had been inhibited germinated when exposed to ambient conditions. These findings suggest that flat sour spoilage of thermal-processed foods could be avoided by incorporating CO_2 at low pressure (50 psi) in the package.

Spore germination was impaired in carbonated AAMS broth. However, the addition of 50 mM $NaHCO_3$ to AAMS broth enhanced rather than impaired germination and outgrowth.

An attempt was made to characterize the nature of the lesion inflicted by CO_2 on vegetative cells. There was no evidence of a difference in DNA/RNA leakage indicated by analysis of the ultraviolet absorption spectra of culture supernates of CO_2 treated and control cells.

REFERENCES

1. Baker, R. C., R. A. Quereshi and J. H. Hotchkiss. 1986. Effect of an elevated level of carbon dioxide containing atmosphere on the growth of spoilage and pathogenic bacteria at 2°, 5°, and 13°C. Poult. Sci. 65: 729-737.

2. Bergey's Manual of Determinative Bacteriology. 8th edition, 1984. The Williams and Wilkins Company, Baltimore, MD., pp. 529-540.

3. Blickstad, E., S. O. Enfors, and G. Molin. 1981. Effect of hyperbaric carbon dioxide pressure on the microbial flora of pork stored at 4° or 14°C. J. Appl. Bacteriol. 50: 493-504.

4. Blocher, J. C., and F. F. Busta. 1983. Bacterial spore resistance to acid. Food Technol. 37(11): 87-99.

5. Cross, G., J. Wolf and A. N. Barker. 1973. The effect of sodium bicarbonate on the germination of spores of some Bacillus species, pp 199-205. In A. N. Barker G. W. Gould and J. Wolf (ed.), Spore Research, Academic Press, New York.

6. Daniels, J. A., R. Krishnamurthi and S. Rizvi. 1985. A review of effect of carbon dioxide on microbial growth and food quality. J. Food Protection 48: (6) 532-537.

7. Dixon, N. M., and D. B. Kell. 1989. A Review. The inhibition by CO_2 of the growth and metabolism of micro-organisms. J. Appl. Bacteriol. 67: 109-136.

8. Doyle, M. P. 1983. Effect of carbon dioxide on toxin production by Clostridium botulinum. Eur. J. Appl. Microbiol. Biotechnol. 17: 53-56.

9. El-Mabsout, Y. E., and K. E. Stevenson. 1979. Activation of Bacillus stearothermophilus spores at low pH. J. Food Sc. 44: 705-709.

10. Enfors, S. O., and G. Molin. 1978. The influence of high concentration of carbon dioxide on the germination of bacterial spores. J. Appl. Bacteriol. 45: 279-285.

11. Enfors, S. O., and G. Molin. 1978. Mechanism of the inhibition of spore germination by inert gases and carbon dioxide. In Spores VII, ed. Chambliss, G. & Vary, J.C. pp. 80-84. Washington: Society of Microbiology.

12. Enfors, S. O., and G. Molin. 1980. Effect of high concentrations of carbon dioxide on the growth rate of Pseudomonas fragi, Bacillus cereus, and Streptococcus cremoris. J. Appl. Bacteriol. 48: 409-416.

13. Feeherry, F. E., Munsey, D. T., and D. B. Rowley. 1987. Thermal inactivation and injury of Bacillus stearothermophilus spores. Appl. and Environ. Microbiol. 53: 365-367.

14. Jay, J. M. 1986. Characteristics and growth of thermophilic microorganisms. Ch. 24. In "Modern Food Microbiology," J. M. Jay (Ed.), p. 593. Van Nostrand Reinhold Co., New York, NY.

15. Haas, G. J., H. E. Prescott, E. Dudley, R. Dik, C. Hintlian, and L. Keane. 1989. Inactivation of microorganisms by carbon dioxide under pressure. J. Food Safety. 9: 253-265.

16. Hachisuka, Y., N. Kato, and N. Asano. 1956. Inhibitory effect of sodium carbonate on spore germination of Bacillus subtilis. J. Bacteriology. 71: 250-251.

17. Madden, R. H. and B. Moss , 1987. Extension of shelf life of minced beef by storage in vacuum packages with carbon dioxide. J. Food Protect. 50: 229-233.

18. Molin, G. 1983. The resistance to carbon dioxide of some food related bacteria. Eur. J. Appl. Microbiol. Biotechnol. 18: 214-217.

19. Shigehisa, T., T. Ohmori, A. Saito, S. Taji and R. Hayashi. 1991. Effects of high hydrostatic pressure on characteristics of pork slurries and inactivation of microorganisms associated with meat and meat products. Int. J. Food Microbiol. 12: 207-216.

20. Silliker, J. H. The influence of atmospheres containing elevated levels of CO_2 on growth of psychrotrophic organisms in meat and poultry. Ch. 37. 370-375.

21. Thomas, Y. O., Kraft, A. A., Rust, R. E., and Hotchkiss, D. K. 1984. Effect of carbon dioxide flushing and packaging methods on the microbiology of packaged chickens. J. Food Sci. 49: 1367-1371.

22. Widdowson, J. P. 1967. Bacillus pantothenticus spores activated by dimethylformamide and dimethylsulphoxide. Nature. 214: 812-813.